

Synthesis of new C⁶ substituted peptidyl nucleosides. A mechanistic study

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Abstract: The synthesis of four new peptidyl nucleosides is reported. The kinetic data obtained for the transesterification of 2'/3'-O-benzyloxycarbonyl-L-*p*-nitrophenylalanyl 5'-O-trityl ribonucleosides which contain different substituents at C⁶ position of the purine residue indicates for different mechanisms of the transesterification reaction. The peptidyl nucleosides with an amino group at C⁶ position and those lacking such group at that position have two distinctive mechanisms of transesterification.

Keywords: Nucleosides; transesterification; tautomerization.

1. Introduction

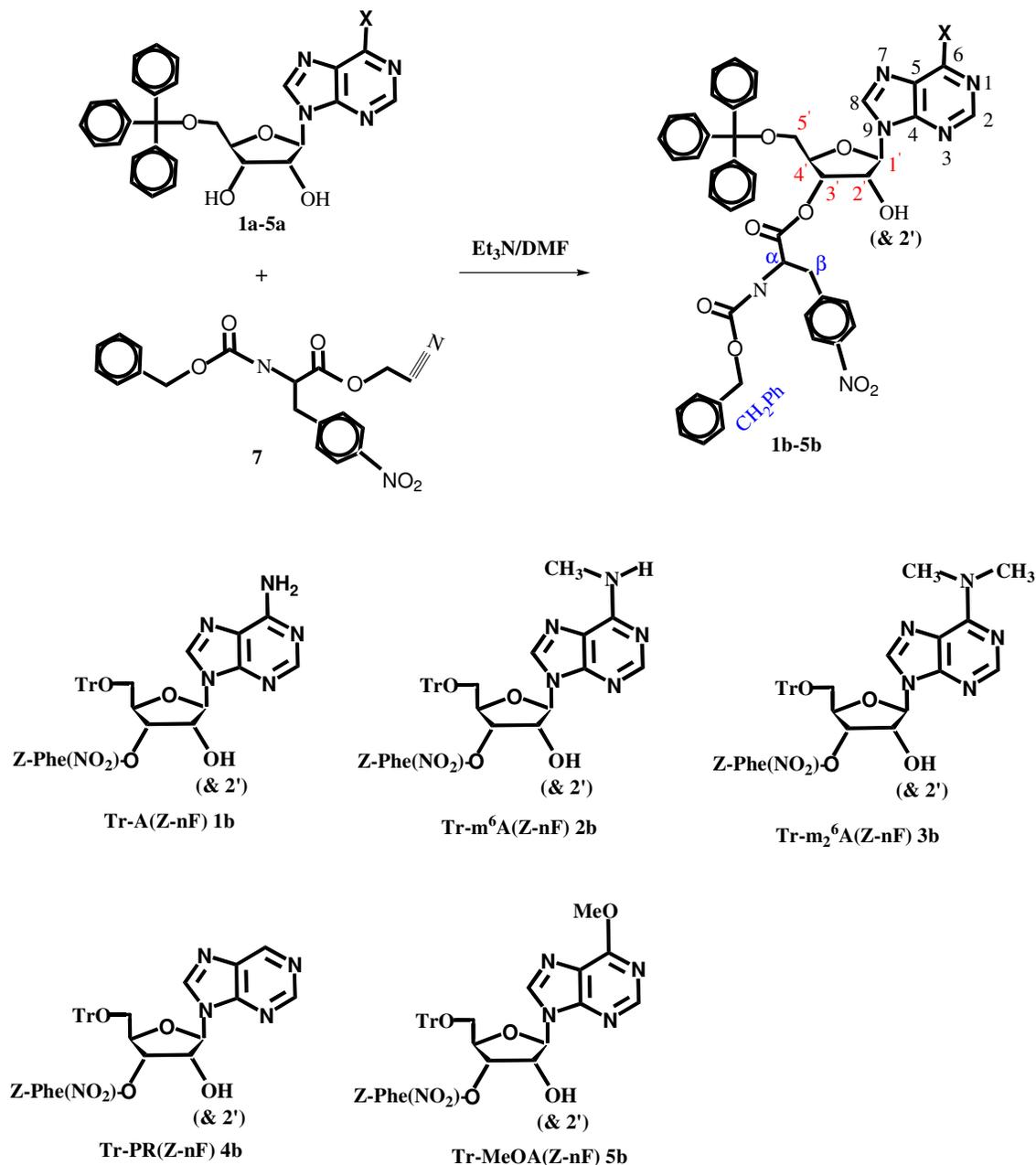
The 3'-CCA sequence of tRNA is a universal ligand for protein biosynthesis; it is recognized by aminoacyl-tRNA synthetases, EF-Tu, and 23 S rRNA.^{1,2} Experiments using in vitro transcribed tRNA^{Val} variants demonstrated the importance of the 3'-CCA sequence for aminoacylation^{3,4} and its significance in formation of the ternary complex between Val-tRNA, EF-Tu, and GTP⁵. On ribosomes, the CCA end of tRNA interacts with 23 S rRNA at all ribosomal tRNA binding sites⁶. The importance of the CCA end in ribosome-catalyzed peptide bond formation is well established^{1,7}. Chemically synthesized aminoacyl oligonucleotides have been used to demonstrate the significance of the 3'-CCA sequence as a peptide acceptor during peptide bond formation on ribosomes.⁸ *E. coli* tRNA^{Val} with mutations in the 3'-CCA sequence inhibits the peptidyltransferase activity of the ribosome.⁹ Although, the importance of the CCA end in ribosome-catalyzed peptide bond formation is well established, there are many questions that remain unclear. Why this sequence ends with A and how nucleobase affects the reactivity of ribonucleoside 2'/3'-OH group which plays essential role in ribosome catalyzed peptide bond formation¹⁰ are some of them. We synthesized 2'/3'-O-aminoacyl nucleosides with different substitutions at C-6 position of the purine base in order to probe their effect on the 2'/3'-OH group reactivity during external acyl group transfer (ethanolysis).

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2. Results and Discussion

It is known that the acidity of 2'/3'-OH group in ribonucleosides is steered by the nucleobase structure¹¹. In our previous study¹² we carried out modifications including atomic and group substitutions in the adenine-9-yl group of adenosine in order to obtain adenosine derivatives with varying 2'/3'-OH pKa values.



Scheme 1. Synthesis of 2'/3'-O-benzyloxycarbonyl-L-p-nitrophenylalanyl 5'-O-trityl adenosine derivatives **1b-5b**. The 5'-O-tritylated ribonucleosides **1a-5a** were synthesized according to the literature^{13,14} and were amidoacylated with benzoyloxycarbonyl-L-p-nitrophenylalanyl cyanomethyl ester **6**¹⁵. Z = benzyloxycarbonyl group; Tr = trityl group.

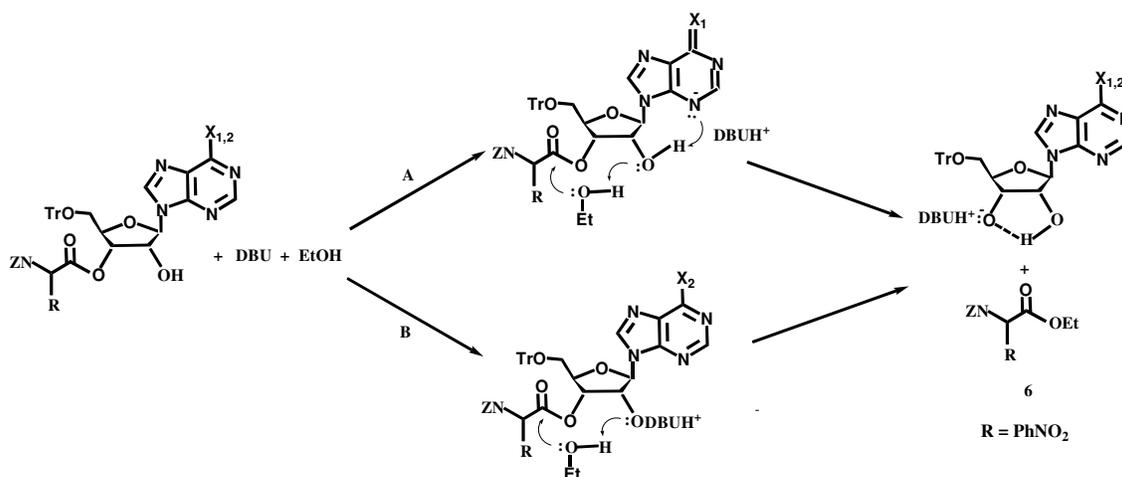
We showed that the apparent rate constants of the external transesterification (ethanolysis) of the corresponding 2'/3'-O-benzyloxycarbonyl-L-*p*-nitrophenylalanyl 5'-O-trityl adenosine derivatives strongly depend on the presence of both a free 2'/3'-OH group and a nucleobase as well as on the nucleobase structure integrity. Further, we demonstrated that nucleobase affects 2'/3'-OH acidity through C1'-C2'-bond inductive effect as well as the slope of the Brønsted plot obtained for the ethanolysis of the substrates demonstrated that 2'/3'-OH group is a general base catalyst of the external acyl group transfer (ethanolysis). Here we carried out modifications including atomic and group substitutions at C⁶ position of the adenine-9-yl group in order to study the mechanism and to probe the possible remote effect of the substituents on the 2'/3'-OH group catalytic activity. For this purpose, five ribofuranosyl-purines have been synthesized with different substituents at C⁶ position of the adenyl residue. After tritilation of 5'-OH group they have been aminoacylated with benzyloxycarbonyl-L-*p*-nitrophenylalanyl cyanomethyl ester to obtain the 2'/3'-O-benzyloxycarbonyl-L-*p*-nitrophenylalanyl 5'-O-trityl ribofuranosyl-purine derivatives **1b-5b** (Scheme 1). External transesterification is known to be catalyzed by the ribosome,¹⁶ in which the P-site

Table 1. Kinetic data for the ethanolysis of 2'/3'-O-benzyloxycarbonyl-L-*p*-nitrophenylalanyl 5'-O-trityl ribonucleosides **1b-5b** in acetonitrile at 25°C.

2'/3' -O-aminoacyl nucleosides		$k_{\text{obs.}}(\text{min}^{-1})$
Tr-A(Z-nF)	1b	656.9±60
Tr-m ⁶ A(Z-nF)	2b	375.2±20
Tr-m ₂ ⁶ A(Z-nF)	3b	308.4±25
Tr-PR(Z-nF)	4b	356.3±30
Tr-MeOA(Z-nF)	5b	338.4±25

excludes water until the moment when release factors interact with a stop codon¹⁷. To mimic the environment of this site we studied the ethanolysis of the substrates **1b-5b** in the aprotic polar organic solvent acetonitrile using the strong, bulky organic base 1,8-diazabicyclo[5.4.0]-undec-7-en (DBU) as base catalyst. k_{obs} constants (Table 1) were obtained from the pseudo-first order decrease of the substrate concentration in HPLC chromatograms.

From table 1 is seen that as overall, there is only a slight difference in the rate constants for the ethanolysis of compounds **1b-5b**. However, small but distinct difference (~ 2 fold) in k_{obs} constants exists between substrate **1b** and the rest four substrates **2b-5b**. 2'/3'-O-benzyloxycarbonyl-L-*p*-nitrophenylalanyl 5'-O-trityl ribonucleoside derivatives **1b-5b** do not react with ethanol in the absence of a strong base, such as DBU and the latter suggests that DBU promotes the transesterification reaction (Scheme 2). There are two important sites for proton abstraction in **1b, 2b**, which are the C-6 amino groups and 2'/3'-OH group. Only one site in **3b-5b** exists and that is the 2'/3'-OH group. Deprotonation of the second reagent, ethanol by DBU could take place as well. Considering the pKa of the sites for proton abstraction and the kinetic data that we obtained for the ethanolysis reaction, it is possible to distinguish two different mechanisms through which the transesterification of **1b, 2b** and **3b-5b** proceeds (Scheme 2). pKa of the amino group of adenosine is very acidic while 2'/3'-OH group has pKa of 12.17¹¹ and the pKa of ethanol is 16. Due to the much lower pKa values of ionization of the amino groups of **1b, 2b** at C-6 position the concentration of the 6-nitrations is much higher than those of the 2'/3'-oxyanions. Moreover, we assume a tautomerization¹¹ of these nitrations to 3-nitrations (Scheme 2, mechanism A). The formation of a 3-nitranion leads to hydrogen bonding between the 2'-OH group and the adenine-9-yl N3. This hydrogen bond is favored by the low



Scheme 2. Proposed mechanisms of transesterification in the presence of 1,8-diazabicyclo [5.4.0]-undec-7-en (DBU)^{12,18}. X₁ = NH₂ (**1b**), NHCH₃ (**2b**); X₂ = N(CH₃)₂ (**3b**), H (**4b**), MeO (**5b**). pK_a of DBU in acetonitrile is 23.9 pKa units¹⁹. Products of the reaction have been confirmed by HPLC analytical runs of available standards.

dielectric acetonitrile medium and generates enhanced electron density on the 2'-OH group and by this way increasing its reactivity during the ethanolysis which results in higher (~2-fold) k_{obs} for **1b**. Similar possibility of tautomerization exists for substrate **2b**, however, it has at C⁶ position a secondary amine which results in a lower concentration of the 3-nitranion compared to that of **1b**. This is likely to be the reason for the lower k_{obs} constant for **2b** (Table 1) in addition to the steric hindrance. The deprotonation of the amino groups by DBU and the tautomerization probably takes place in a fast pre-equilibrium step due to the sharp differences in the pK_a values of the C-6 amino groups and the DBU, and the thermodynamically favorable direction of the proton transfer. However, the rate determining step is the attack of the EtOH which is facilitated by the 2'/3'-OH group as it is already hydrogen bonded to the 3-nitranion. This kind of tautomerization is not possible for the rest three compounds (**3b-5b**), as they do not have amino groups (Scheme 1). Their lower rate constants compared with the value of the k_{obs} constant for **1b** could be explained only with a different mechanism (Scheme 2, mechanism B) where DBU deprotonates 2'/3'-OH group rather than EtOH (the second reagent) because of its significantly lower pK_a value of ionization than that of EtOH. Deprotonation of the 2'/3'-OH group occurs again in a pre-equilibrium step and the 2'/3'-oxyanion facilitates the attack of the EtOH at the rate-determining transition state. Using kinetic isotope effects and linear free energy relationships we have demonstrated in our previous¹⁸ study that the proton from EtOH has been almost entirely transferred to the 2'/3'-oxyanion in a late asymmetric rate-determining transition state. The latter mechanism has already been studied in detail^{12,18} for similar compounds. However, the common step for both mechanisms described above is the rate-determining step which is the attack of the nucleophile since the nucleofuge is less basic and is a better leaving group than the attacking nucleophile.

3. Conclusion

The kinetic data (Table 1) obtained for the ethanolysis of 2'/3'-O-benzyloxycarbonyl-L-*p*-nitrophenylalanyl 5'-O-trityl adenosines **1b-5b** shows that the type of the substituent at C-6 position of purine residue does not affect substantially the rate of the ethanolysis i.e. the activity of 2'/3'-OH group. However, the data is suggestive of different mechanisms of transesterification for peptidyl nucleosides with an amino group at C⁶ position and those lacking amino group at that position.

4. Experimental

All solvents were distilled before use. Thin layer chromatography (TLC): aluminium sheets precoated with silica gel 60 F₂₅₄ (Merck). Column chromatography was carried out by using silica gel 60 (230-400 mesh, Merck) and Dowex (OH⁻ form). Reverse phase HPLC analyses were performed on Waters Liquid Chromatograph equipped with absorbance detector model 441 set at 254 nm and column Nucleosil 100-5C18 (12.5 cm X 4.6mm) for analytical runs, or Nucleosil 100-5C₁₈ (25 cm × 10 mm) for semi-preparative runs. ¹H spectra were taken on a BRUKER Avance-DRX 250 spectrometer at 300 K with tetramethylsilane as internal standard. Chemical shifts are reported in δ (ppm). The following starting materials were used (commercially available or prepared according to the literature): adenosine, nebularine, TrCl, DBU (Flucka); 2',3',5'-tri-O-acetyladenosine²⁰; 1,2-bis[(dimethylamino)methylene]hydrazine dihydrochloride, 9-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)-6-(1,2,4-triazol-4-yl)purine, 6-methoxy-9-(β-D-ribofuranosyl)-purine, 9-(β-D-ribofuranosyl)-6-(dimethylamino)-purine and 9-(β-D-ribofuranosyl)-6-(monomethylamino)-purine were prepared according to the literature¹³. All nucleosides were tritilated using previously described protocols¹⁴. benzyloxycarbonyl-L-*p*-nitrophenylalanyl cyanomethyl ester and benzyloxycarbonyl-L-*p*-nitrophenylalanyl ethyl ester were prepared according to the literature¹⁵.

4.1 General procedure for synthesis of compounds (1b-5b):

Ten equivalents triethylamine and 1.2 equivalents of benzyloxycarbonyl-L-*p*-nitrophenylalanyl cyanomethyl ester were added to a 0.1 M solution of 5'-O-trityl nucleoside and stirred at 25 °C overnight. The reaction mixture was evaporated to dryness under reduced pressure, dissolved in acetonitrile and applied on a Nucleosil 100-5C₁₈ (25 cm × 10 mm) semi-preparative column. The aminoacylated nucleosides were eluted isocratically with appropriate buffer for each substrate. The analytically pure fractions were pooled and evaporated immediately. Total yields of the isolated products were in the range of 60-70%. They were 2:1 isomeric mixture of the 3' and 2' positional isomers. ¹H NMR were taken on the isomeric mixtures, the integral of the anomeric proton at 5.4-5.8 ppm was assigned as unity which gave integrals for the protons from the ribose residue of the 3' and 2' positional isomers of 0.6 H and 0.4 H respectively.

2'/3'-O-benzyloxycarbonyl-L-*p*-nitrophenylalanyl 5'-O-trityl adenosine¹² (1b):

The ester has been isolated with HPLC using Nucleosil 100-5C₁₈ 250x10 mm column and mobile phase 50% acetonitrile, 50% 0.02M KH₂PO₄/K₂HPO₄ pH=7.0 flow rate 1ml/min (Rt=45min). yield: 58%; ¹H NMR (CDCl₃, 25 °C, 250 MHz): δ = 3.22 (2H, βCH₂), 3.48 (2H, H-5'), 4.26 (0.6H, H-4' from 3'-isomer), 4.37 (0.4H, H-4' from 2'-isomer), 4.75 (1.4H, αCH, H-2' from 2'-isomer), 5.01 (0.6H, H-2' from 3'-isomer), 5.08 (s, 2H, CH₂Ph), 5.50 (0.4H, H-3' from 2'-isomer), 5.69 (0.6H, H-3' from 3'-isomer) 5.86 (d, J_{1',2'} = 6.6 Hz, 1H, H-1'), 7.22-7.40 (m, 22H, C₆H₅, NH₂), 8.03 (m, 4H, C₆H₄), 8.09 (s, 1H, H-2), 8.27 (s, 1H, H-8);

2'/3'-O-benzyloxycarbonyl-L-*p*-nitrophenylalanyl-N⁶-monomethyl-5'-O-trityl adenosine (2b):

The ester has been purified by HPLC using Nucleosil 100-5C₁₈ 250x10 mm column and mobile phase 65% acetonitrile, 35% 0.02M KH₂PO₄/K₂HPO₄, pH=7.0 flow rate 1ml/min (Rt=26min). yield: 80%; ¹H NMR (CDCl₃, 25 °C, 250 MHz): δ = 3.10-3.45 (10H, βCH₂, H-5', NCH₃), 4.15 (1H, H-4'), 4.61 (1H, αCH), 4.85 (1H, H-2'), 5.0 (s, 2H, CH₂Ph), 5.12 (1H, HN), 5.32 (1H, H-3'), 5.64 (d, J_{1',2'} = 6.4 Hz, 1H, H-1'), 7.0-7.44 (m, 22H, C₆H₅, C₆H₄), 7.85 (s, 1H, H-2), 8.10 (2H, C₆H₄), 8.2 (s, 1H, H-8);

2'/3'-O-benzyloxycarbonyl-L-p-nitrophenylalanyl-N⁶,N⁶-dimethyl-5'-O-trityl-adenosine (3b):

The compound has been isolated with HPLC using Nucleosil 100-5C₁₈ 250x10 mm column and mobile phase 65% acetonitrile, 35% 0.02M KH₂PO₄/K₂HPO₄, pH=7.0 flow rate 1ml/min (Rt=30min). yield: 61%; ¹H NMR (CDCl₃, 25 °C, 250 MHz): δ = 3.17-3.58 (10H, βCH₂, H-5', N(CH₃)₂), 4.26 (1H, H-4'), 4.75 (1H, αCH), 4.92 (1H, H-2'), 5.08 (s, 2H, CH₂Ph), 5.38 (1H, HN), 5.48 (1H, H-3'), 5.76 (d, J_{1',2'} = 6.8 Hz, 1H, H-1'), 7.07-7.44 (m, 22H, C₆H₅, C₆H₄), 7.97 (s, 1H, H-2), 8.12 (2H, C₆H₄), 8.24 (s, 1H, H-8);

2'/3'-O-benzyloxycarbonyl-L-p-nitrophenylalanyl 5'-O-trityl-nebularine (4b):

4b has been purified by HPLC using Nucleosil 100-5C₁₈ 250x10 mm column and mobile phase 60% acetonitrile, 40% 0.02M KH₂PO₄/K₂HPO₄, pH=7.0 flow rate 1ml/min (Rt=28min). yield: 59%; ¹H NMR (CDCl₃, 25 °C, 250 MHz): δ = 2.72-3.29 (4H, βCH₂, H-5'), 3.61-4.21 (3H, H-4', H-3', H-2'), 4.61 (1H, αCH), 5.03 (s, 2H, CH₂Ph), 5.07 (1H, HN), 5.43 (d, J_{1',2'} = 6.4 Hz, 1H, H-1'), 7.03-7.34 (m, 22H, C₆H₅, C₆H₄), 7.96 (m, 2H, C₆H₄), 8.06 (s, 1H, H-2), 8.85 (s, 1H, H-8), 9.12 (s, 1H, H-6);

2'/3'-O-benzyloxycarbonyl-L-p-nitrophenylalanyl-6-methoxy-5'-O-trityl-nebularine (5b):

The ester has been isolated with HPLC using Nucleosil 100-5C₁₈ 250x10 mm column and mobile phase 60% acetonitrile, 40% 0.02M KH₂PO₄/K₂HPO₄, pH=7.0 flow rate 1ml/min (Rt=27min). yield: 63%; ¹H NMR (CDCl₃, 25 °C, 250 MHz): δ = 1.93-3.27 (7H, βCH₂, H-5', OCH₃), 3.62-4.26 (3H, H-4', H-3', H-2'), 4.69 (1H, αCH), 5.04 (s, 2H, CH₂Ph), 5.72 (d, J_{1',2'} = 6.6 Hz, 1H, H-1'), 7.19-7.25 (m, 24H, C₆H₅, C₆H₄), 8.06 (s, 1H, H-2), 8.39 (s, 1H, H-8);

N-benzyloxycarbonyl-L-p-nitrophenylalanine ethyl ester (6, Scheme 2):

¹H NMR (250 MHz, CDCl₃, 25° C, TMS): δ = 1.10 (m, 3H, CH₃), 1.41 (m, 2H, CH₂), 3.14 (d, J_{a,b} = 6.3 Hz, 1H, βCH), 3.30 (d, J_{a,b} = 5.2 Hz, 1H, βCH), 4.72 (3H, αCH and CH₂CN), 5.11 (s, 2H, CH₂Ph), 5.18 (d, J = 7.5 Hz, 1H, NH), 7.26-7.44 (m, 7H, C₆H₅ and C₆H₄), 8.18 (2H, C₆H₄); Analytical RP-HPLC, column Nucleosil 100-5C₁₈ (12.5 cm X 4.6mm), 65% CH₃CN in 0.02 KH₂PO₄/K₂HPO₄ buffer, pH 7.0; flow rate, 1.0, 298.2K) Rt = 6.5 min.

4.2 A typical kinetic experiment: To 100 μL of 0.0152 M solution of the 2'/3'-O-peptidyl adenosine derivative **1b-5b** in dry acetonitrile were added 31.9 μL dry acetonitrile, 10 μL 1.52 M ethanol solution in dry acetonitrile and 10.12 μL, 0.15M solution of DBU in dry acetonitrile. Aliquots were withdrawn at appropriate time intervals, diluted with mobile phase and subjected to RP-HPLC analysis with isocratic elution with 55-68% acetonitrile (depending on adenosine derivative) in 20mM K₂HPO₄/KH₂PO₄ buffer, pH 7.0), 1 mL/min flow rate at 298.2 K. The concentrations of the substrate and the reaction products were calculated from their peak areas. Each rate constant k_{obs} was calculated from kinetic data with Grafit 4.0 and the average value was taken from more than three experiments.

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